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Isolation and Characterization of Neural Progenitor Cells From Post-Mortem Human Cortex

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Post-mortem human brain tissue represents a vast potential source of neural progenitor cells for use in basic research as well as therapeutic applications. Here we describe five human neural progenitor cell cultures derived from cortical tissue harvested from premature infants. Time-lapse videomicrography of the passaged cultures revealed them to be highly dynamic, with high motility and extensive, evanescent intercellular contacts. Karyotyping revealed normal chromosomal complements. Prior to differentiation, most of the cells were nestin, Sox2, vimentin, and/or GFAP positive, and a subpopulation was doublecortin positive. Multilineage potential of these cells was demonstrated after differentiation, with some subpopulations of cells expressing the neuronal markers β-tubulin, MAP2ab, NeuN, FMRP, and Tau and others expressing the oligodendroglial marker O1. Still other cells expressed the classic glial marker glial fibrillary acidic protein (GFAP). RT-PCR confirmed nestin, SOX2, GFAP, and doublecortin expression and also showed epidermal growth factor receptor and nucleostemin expression during the expansion phase. Flow cytometry showed high levels of the neural stem cell markers CD133, CD44, CD81, CD184, CD90, and CD29. CD133 markedly decreased in high-passage, lineage-restricted cultures. Electrophysiological analysis after differentiation demonstrated that the majority of cells with neuronal morphology expressed voltage-gated sodium and potassium currents. These data suggest that post-mortem human brain tissue is an important source of neural progenitor cells that will be useful for analysis of neural differentiation and for transplantation studies.

Key words: stem cells; cadavers; tissue engineering; cells, cultured; cell differentiation

Cultured neural progenitor cells hold considerable promise both in terms of their application to a wide variety of research paradigms and in the development of potential therapeutic modalities (Bjorklund and Lindvall, 2000). In the case of human neural progenitor cells (hNPCs), the primary source has been donated fetal tissue (Flax et al., 1998; Svendsen et al., 1999; Tamaki et al., 2002; Vescovi et al., 1999a). However, the post-mortem brain represents a potentially vast supply of hNPCs that could reduce or eliminate reliance on fetal or embryonic sources. The practicality of such an approach is supported by recent work demonstrating the viability of hNPCs obtained from cadaveric donors, even after post-mortem intervals exceeding 20 hr (Palmer et al., 2001).

NPCs (variously referred to as neural stem cells or multipotent neural precursors) comprise a relatively undifferentiated population of cells capable of giving rise to the broad array of specialized neurons and glia of the central nervous system (CNS; Gage, 1998; McKay, 1997; Temple, 1999). Long assumed to be exclusive to the developing CNS, these cells have now been shown to persist in adult mammals, including humans (Cameron and McKay, 1998; Chiasson et al., 1999; Palmer et al., 1997; Pincus et al., 1997; Temple, 1999). Research showing that NPCs can be isolated, cultured, expanded, and transplanted has raised the possibility of their use as donor cells for the repair of CNS injuries and diseases. Furthermore, the availability of cultured NPCs allows detailed study of the mechanisms of neural differentiation and the genetic and environmental signals that direct phenotypic special-

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ization in the mammalian CNS (Gritti et al., 1996; Lendahl et al., 1990; Takahashi et al., 1999).

In previous work, we have described methodological advances that allow the routine purification of hNPCs from surgical specimens, biopsy material, and post-mortem tissues (Palmer et al., 2001). Under ideal conditions, precursors from postnatal and adult brain can be exponentially expanded as monolayer cultures, cryopreserved, and recultured for up to 40 population doublings prior to reaching senescence. In one instance, proliferative precursors were isolated and expanded from multiple brain regions at more than 20 hr post-mortem (Palmer et al., 2001). In addition, similar cultures could be initiated from cryopreserved post-mortem tissues with only moderate losses in cell recovery (Palmer et al., 2001). The fact that human precursors were still viable after such long post-mortem intervals suggests that neural precursors are relatively resistant to post-mortem ischemic and oxidative stress compared with neurons (Safar, 1979).

The experiments described here provide a broad initial assessment of postnatal hNPCs isolated from post-mortem tissue. Because previous data demonstrated greater proliferative and cell-lineage potential in younger brains (Palmer et al., 2001), we deal here exclusively with tissue taken from premature infants. In addition, by identifying a range of surface markers present on these cells, this work points toward potential enrichment strategies that might enhance the yield of multipotent CNS progenitors during isolation as well as molecular targets for influencing key processes, such as proliferation, migration, immunogenicity, and differentiation.

MATERIALS AND METHODS

Human Subjects

Informed consent for the donation of brain tissue was acquired by the authors prior to tissue acquisition, under the auspices of the protocol for the National Human Neural Stem Cell Resource (NHNSCR) approved by the Children's Hospital of Orange County Institutional Review Board. All tissues were acquired in compliance with NIH and institutional guidelines. Tissues used in the present study were from cases SC22, SC23, SC27, SC29, and SC30.

Pathogen Testing

Heart's blood obtained at autopsy was tested in the Saint Joseph Hospital (Orange, CA) clinical laboratory for the presence of hepatitis B core antigen and surface antibody, hepatitis C antibody, HTLV-I and -II, syphilis RPR, HIV-1 and -2 antibody, cytomegalovirus, and HIV-1 antigen.

Autopsy and Brain Dissection

The autopsy followed standard procedures with the addition of methodology directed toward obtaining a sterile field for the removal and preparation of tissue, albeit within the constraints of an autopsy facility. After removal of the brain with cerebellum and brainstem intact, a midsagittal cut was made to separate the cerebral hemispheres, followed by immersion of the left cerebral hemisphere in 4% neutral buffered formaldehyde (10% formalin) for neuropathological examination. The brainstem and cerebellum were separated from the right hemisphere by transverse section through the cerebral peduncles and rostral to the superior colliculi. A second transverse cut was then made through the brainstem, ~1 cm posterior to the above-described cut, resulting in "section A" (Fig. 1). The cerebellum was sectioned parasagittally, just lateral to the most lateral extent of the brainstem, and then again 1 cm lateral to that, the second cut giving "section B" (Fig. 1). The right cerebral hemisphere was then placed medial surface down, and coronal sections were made at 1-cm intervals from frontal to occipital poles. The coronal section just posterior to the most anterior extent of the temporal lobes is "section D" (Fig. 1). The section 2 cm posterior to that, and 3 cm posterior to the most anterior extent of the temporal lobe, is "section C" (Fig. 1). The following regions were then identified and dissected from the appropriate sections (Fig. 1): 1) substantia nigra (from section A), 2) cerebellar cortex (from section B), 3) hippocampus, 4) centrum semiovale, 5) cortex (from section C), 6) periventricular zone from the head of the caudate nucleus, and 7) caudate nucleus (from section D). Brain region specimens were placed in separate petri dishes and rinsed three times with DGA (see below).

Neuropathology

Standard examination was performed by a neuropathologist and a written report generated, any abnormalities being noted.

Basic Media

The base medium used was a high glucose 1:1 DMEM: F12 (Irvine Scientific, Irvine, CA). The basal medium used for all other media (DGA) consisted of base medium containing 292 μg/ml glutamine (Irvine Scientific), 100 U/ml penicillin (Irvine Scientific), 100 μg/ml streptomycin (Irvine Scientific), 50 μg/ml gentamicin (Sigma, St. Louis, MO), 10 μg/ml ciprofloxacin (Bayer), and 2.5 μg/ml amphotericin (Gibco, Grand Island, NY). Medium used for all washes and for glial cell culture (DGF) consisted of DGA containing 10% fetal bovine serum (FBS; defined, heat-inactivated; Hyclone, Logan, UT).

Cell Isolation

All procedures were performed in sterile fashion in a class II biosafety cabinet. Tissue was diced with scalpel blades and incubated at 37°C for 20–40 minutes in DGA containing 2.5 U/ml papain (Worthington, Freehold, NJ), 250 U/ml DNase I (Worthington), and 1 U/ml neutral protease (Roche, Indianapolis, IN). Partially digested tissue was further dissociated by trituration and three washes in DGF with centrifugation. Whole tissue dissociates were plated directly.

Glial Cultures

Glial cultures were established, with a modification of the method of De Groot et al. (1997), by plating crude homogenates onto uncoated plastic culture dishes (100 mm, tissue culture-treated; TPP) in DGF at a ratio of approximately 300 mg wet weight of starting material per 25 cm^2 of surface area. Cultures were fed weekly by replacing 100% of the medium with fresh DGF and then more often (up to daily) as they approached confluence. Near confluence, medium for some cultures was

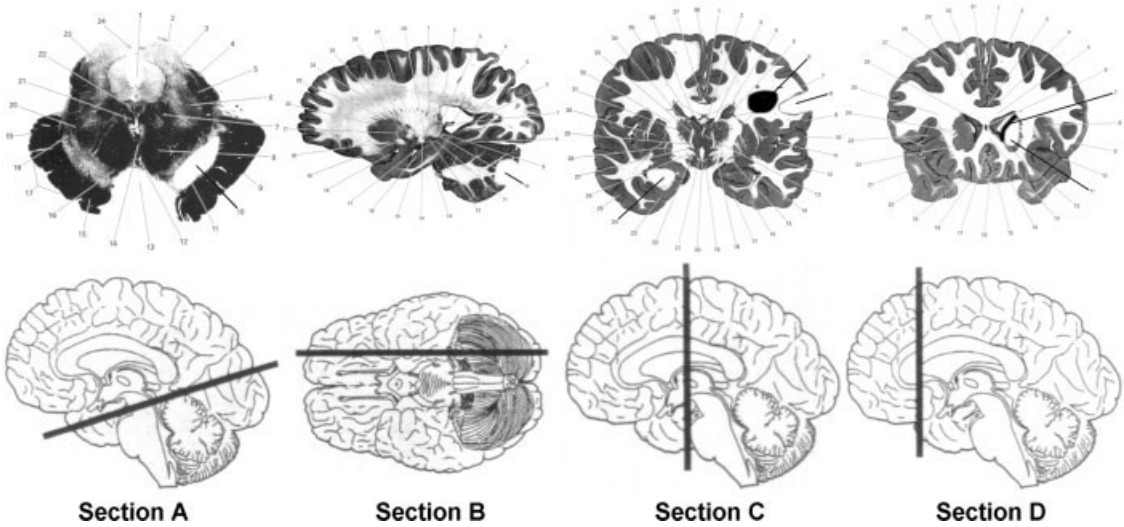

Fig. 1. Brain areas sampled for culture and cryopreservation. Section A: The substantia nigra, lying in the ventral midbrain just dorsal to the cerebral peduncle, is shown as the white area indicated by line 10 in "section A." This tissue was taken just caudal to the transverse cut made to separate the brainstem and cerebellum from the cerebral hemispheres. Section B: The cerebellar cortex is shown by the white area indicated by line 10 in "section B." This sample was taken from the lateral sagittal section of the cerebellum. Section C: 1) Hippocampal formation (shown on left for clarity), 2) centrum semiovale (shown as blackened area, sparing gray matter), and 3) cortex (sparing white matter) from the areas shown by lines 24, 6, and 8, respectively, in "section C." These samples were taken from the third section posterior to the anterior-most extent of the temporal lobe. Section D: 1) Periventricular zone, adjacent to the head of the caudate nucleus (2–3 mm thick), and 2) caudate nucleus, from areas shown by lines 7 and 11, respectively, in "section D." These samples were taken from the first section posterior to the anterior-most extent of the temporal lobe. (Pictures and drawings with permission from: J. Hanaway et al., The brain atlas—a visual guide to the human central nervous system, Fitzgerald Science, Inc., Bethesda, MD, 1998, p 50, 62, 116, 152.)

replaced with Neurobasal medium (Gibco) with B-27 supplement (Gibco). After 24 hr, this medium was removed and collected, and fresh DGF was added to the culture. The collected medium served as the glial-conditioned medium (GCM) component of the differentiation medium described below. Glial cultures were cycled through the DGF and Neurobasal media to provide GCM for up to 10 cycles before the cultures were lifted and cryopreserved as described below.

NPC Cultures

Crude homogenates were initially plated onto fibronectin-coated dishes (60 mm, tissue culture-treated; Cell-Star) in primary growth medium (PGM) composed of DGF containing 10% BIT 9500 (Stem Cell Technologies), 40 ng/ml basic fibroblast growth factor (FGF-2; Invitrogen, La Jolla, CA), 20 ng/ml epidermal growth factor (EGF; Invitrogen), and 20 ng/ml platelet-derived growth factor-AB (PDGF-AB; Peprotech). Dishes had been previously incubated with 200 $\mu l/cm^2$ of fibronectin (5 μg/ml; Sigma) overnight at 37°C, the fibronectin solution was aspirated, and the dishes were allowed to air dry before the introduction of tissue homogenates. In a typical case, approximately 3 g fresh frontoparietal cortex were subjected to mincing and enzymatic digestion, and the resulting crude tissue homogenate was plated into 10 × 60-mm fibronectin-coated plastic Petri dishes.

Twenty-four hours after plating, all nonadherent cells and debris were transferred to a new set of fibronectin-coated culture dishes of the same number and size. The nonadherent cells and debris were pelleted by centrifugation and introduced into the new dishes with fresh growth medium (GM), defined as PGM without the FBS. The adherent cultures were fed with fresh GM. Thereafter, 50% of the medium of all cultures was replaced, daily, with fresh GM. After 10 days in culture, the dishes were agitated by gentle tilting, and 100% of the culture medium was removed. Fifty percent of the volume was replaced with fresh GM, and the removed medium (conditioned GM; CGM) was centrifuged to pellet cell debris and nonadherent cells; 50% by volume of the resulting supernatant was then returned to the dishes.

Secondary Cultures

At near confluence, cultures were passaged by washing them once with Ca^{2+}/Mg^{2+}-free Hanks buffered salt solution (Irvine Scientific) and then lifting with cell dissociation buffer (Gibco). The cells were resuspended in 1:1 CGM:GM and plated onto twice the surface area from which they were lifted. For immunocytochemistry, cells were plated onto fibronectin-coated eight-well glass chamber slides (Lab-Tek).

Cryopreservation

For cryopreservation, lifted cells or minced tissues were suspending in CGM supplemented with 10% dimethylsulfoxide (DMSO). Samples were gradually (~1°C/min) cooled to −80°C in isopropanol freezing containers. Frozen samples were then transferred to liquid nitrogen Dewars for long-term storage at −196°C.

Thawing of vials was accomplished by shaking under warm tap water until the moment when the contents had just liquefied. The contents were then transferred to a centrifuge tube and 10 volumes of DGF added (drop-wise for the initial 2 volumes), with frequent mixing. Resuspended cells were then centrifuged and washed twice with DGF before plating as described above.

Videomicrography

Phase-contrast images of proliferating cultures in 60-mm Petri dishes were taken every 5 min for 24 hr on a Nikon Eclipse TE300 inverted microscope equipped with a Photometrics Cool Snap Fx CCD camera (Roper Scientific). The microscope was enclosed in an opaque polyacrylate chamber, and the environmental conditions within this box were tightly regulated and monitored. Temperature was maintained at 37°C with a heating system engineered by Dr. Seog Woo Rhee (Department of Biomedical Engineering, UCI). Medical-grade 5% CO_2 balanced by air (AirGas) was regulated by a Gilmont Instruments CO_2 sensor and bubbled through 37°C water, then passed into the culture dish via a small aperture in the top of a loose-fitting lid. Phase-contrast images were obtained by using a Nikon Te-PS100 white lamp at the phase I setting. The autofocus setting of an H1222 Nikon Focus Drive was used to maintain appropriate focus throughout the imaging period. The Metamorph (version 5.0r2) multidimensional acquisition software (Universal Imaging Corp.) was used to compile the images into time-lapse video.

Differentiation

Proliferating cultures were differentiated by replacing 50% of the medium, daily, with 1:1 GCM:DGA supplemented with 1% FBS, 100 nM all-*trans*-retinoic acid (Sigma), 20 ng/ml brain-derived neurotrophic factor (BDNF; Chemicon, Temecula, CA), and 20 ng/ml neurotrophin-3 (NT-3; Chemicon). Some cultures were differentiated by replacing 30% of the medium, every 3 days, with B-104-conditioned medium (a kind gift of Dr. Stephen Back, OHSU).

Immunocytochemistry

Cells were fixed for 10 min at room temperature in freshly prepared 4% formaldehyde (from paraformaldehyde) in Dulbecco's phosphate-buffered saline (PBS), pH 7.4. The formaldehyde was removed and replaced with PBS containing 0.5% sodium azide, and the plates were stored at 4°C until analysis. Fixed cells were rinsed in 0.1 mM Tris-buffered saline (TBS), rinsed, blocked in TBS^{++} (0.1 mM TBS, 5% donkey serum, 0.25% Triton X-100) for 1 hr, and incubated overnight with primary antibodies in TBS^{++} at 4°C. Cultures were then rinsed and incubated with fluorescently labeled secondary antibodies (AMCA, Cy2, Cy3, Cy5, or RRX, 1:250; Jackson Immunoresearch, West Grove, PA) overnight at 4°C. Removal of the secondary antibodies was followed by an additional rinse. Some were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) before being rinsed and mounted. Primary antibodies and dilutions were used as follows: nestin (1:1,000; mouse; Pharmingen, San Diego, CA; or 1:100; rabbit; Chemicon), type III β-tubulin (Tuj1; 1:1,000; mouse; Sigma; or 1:100; mouse; Chemicon), MAP2ab (1:250; mouse; Sigma), glial fibrillary acidic protein (GFAP; 1:500; guinea pig; Advance Immuno; or 1:100; rabbit; Chemicon), doublecortin (DCX; 1:100; goat; Santa Cruz Biotechnology, Santa Cruz, CA), tau (1:100; mouse; Chemicon), calbindin D28k (1:500; mouse; Boerhinger Mannheim, Indianapolis, IN), NeuN (1:10; mouse; gift, Mullen Lab; or 1:100; mouse; Chemicon), A2B5 (1:100; mouse; Chemicon), O1 (1:100; mouse; Chemicon), O4 (1:100; mouse; Chemicon), vimentin (1:100; mouse; Sigma), PSA-NCAM (ECAM; 1:100; mouse; Chemicon), and/or FMRP (1:100; mouse; Chemicon). Images were taken on a Bio-Rad confocal system at ×40 or ×63 or an Olympus IX-70 fluorescence microscope at ×4, ×10, ×20, ×40, or ×100.

RT-PCR

Total RNA from hNPCs grown as an adherent monolayer was isolated using a Purescript RNA Isolation Kit (Gentra Systems) and treated with DNA-free (Ambion). Next, RNA was reverse transcribed with MULV. PCR was performed with 3 μl of RT product, 0.75 μl of the forward and back primers (0.5 μg/μl; Qiagen, Chatsworth, CA; Table I), and 0.25 μl of Taq (Amersham, Arlington Heights, IL). All PCR programs were performed on a Techne Genius. PCR products were visualized on 2% agarose gels against a 100-bp ladder.

Flow Cytometry

Cells for flow cytometry were lifted as described above and resuspended in Ca^{2+}/Mg^{2+}-free PBS containing 0.02% sodium azide and 1% human albumin and divided into 100-μl aliquots containing approximately 5×10^5 cells, to which antibodies or isotype controls were added. Manufacturer's suggested concentrations were observed (1:100 for all). Cells were incubated in the dark at room temperature for 20 min and then washed with 2 ml PBS. When the antibody was unconjugated, a secondary antibody (FITC goat anti-mouse, PE sheep anti-mouse, or AMCA donkey anti-mouse) was added and the sample allowed to incubate in the dark for an additional 15 min and then again washed. At the completion of labeling, washed cells were resuspended in 200 μl of PBS containing 7-amino-actinomycin D (7-AAD; 1 μg/ml). All the antibodies used in this study were obtained from BD Pharmingen (name/synonym/clone: CD90/Thy-1/5E10, CD56/NCAM/B159, CD54/ICAM/HA58, CD106/VCAM/51-10C9, CD9/none/M-L13, CD81/TAPA-1/5S-81, CD24/HSA/ML5, CD14/GPI, LPSR/M5E2, CD26/DPPIV/M-A261, CD44/Pgp-1, HCAM/G44-26, CD49f/integrin α6/GoH3, CD15/LeX/MMA, CD29/integrin β1/MAR4, CD45/LCA/HI30, CD130/gp130R/AM64, CD144/cadherin-5/55-7H1, CD166/ALCAM/3A6, CD184/CXCR4/12G5) or Miltenyi (CD34/none/AC136, CD133-1/none/AC133).

Cytometry was performed on a FACS Vantage (BD Biosciences). Color compensation was set using calibrite beads, and samples were optimized for each fluorochrome. Three-color live gating was used to optimize settings and acquire data. Thirty thousand events were collected per sample and stored for analysis.

Transfection

Near-confluent cultures were transfected using Neuroporter Transfection Reagent (Gene Therapy Systems, San Di-

TABLE I. RT-PCR: Primers and Conditions

Gene	Primers 5′–3′	Product size (bp)	Reference for primers (Pubmed ID)	PCR conditions	
				Anneal temp (°C)	No. of cycles
Doublecortin	F–AATCCCAACTGGTCTGTCAAC R–GTTTCCCTTCATGACTCGGCA	405	Custom	59	35
GFAP	F–ACATCGAGATCGCCACCTAC R–ACATCACATCCTTGTGCTCC	219	12020615	64	30
PAX6	F–CCAGCCAGAGCCAGCATGCAGAACA R–GGTTGGTAGACACTGGTGCTGAAACT	950	10891600	73	30
SOX2 (2 sets of primers used; both yielded same results)	First set F–TACCTCTTCCTCCCACTCCA R–ACTCTCCTCTTTTGCACCCC R–ACTCTCCTCTTTTGCACCCC	269	11074522	59	30
	Second set F–GGCAGCTACAGCATGATGCAGGAGC R–CTGGTCATGGAGTTGTACTGCAGG	131	10804179	73	30
Nestin	F–GGCAGCGTTGGAACAGAGGTTGGA R–CTCTAAACTGGAGTGGTCAGGGCT	718	Genebank accession No. AJ270321 (unpublished article)	66	30
EGF-R	F–CGCAAGTGTAAGAAGTGCGAA R–CGTAGCATTTATGGAGAGTGAGTCT	93	11159180	56	30
Nucleostemin	F–CATGACCTGCCATAAGCGGT R–CAATTACTCCAACCCGAATGGC	745	Custom XPrimer online tool	51	30

ego, CA) and the gWiz GFP Transfection Vector. Images were taken on the fluorescence microscope.

Karyotyping

Passage 4 confluent cultures were incubated in GM containing 10 ng/ml colcemid for 18 hr. The cultures were then lifted and centrifuged. Pellets were osmotically shocked with 0.075 M KCl and fixed with 3:1 methanol:glacial acetic acid. Standard cytogenetic analysis was performed by the Genetics Center (Orange, CA).

Electrophysiological Recordings

Whole-cell recordings were made using unpolished glass pipettes with open tip resistances of 3–5 MΩ. Isolated sodium currents were recorded using an internal solution containing (in mM): cesium hydroxide (120), D-gluconic acid (120), NaCl (20), EGTA (1.1), $CaCl_2$ (0.1), $MgCl_2$ (2), HEPES (10), pH 7.2. Potassium currents and action potentials were recorded using an internal solution containing (in mM): potassium gluconate (120), NaCl (20), EGTA (1.1), $CaCl_2$ (0.1), $MgCl_2$ (2), HEPES (10), pH 7.2. The external solution contained (in mM): NaCl (140), KCl (3), $MgCl_2$ (4),$CaCl_2$, (1), HEPES (5), pH 7.2. All data shown are corrected for the 5-mV liquid junction potentials generated in these solutions. Sodium currents were blocked by bath application of 1 μM TTX. Data were acquired with a List EPC7 amplifier, a Digidata 1320A D-A converter (Axon Instruments), a Dell (Dimension 4100) computer, and pCamp8 (Axon Instruments) software. Recordings were made at room temperature.

RESULTS

Human Subject and Pathogen Testing

The patients were 23–25-weeks-of-gestation premature infants who expired shortly after birth to 2 weeks after birth, all from pulmonary complications. No clinical evidence of CNS dysfunction was noted. Parental consent for tissue harvest was obtained after the death of the infants, and tissue harvest was begun within 3 hr. Neither the infants nor the mothers tested positive for any of the pathogens screened. SC22, a male 23-week premature infant, was the oligocythemic maternal twin of a twin-to-twin transfusion who died on his day of birth; SC23 was the polycythemic twin and died on the next day. SC27, a male 23-week premature infant, died of pulmonary complications 2 weeks after birth. SC29 and SC30 were male 25-week premature fraternal twins who died on the day of and the day after birth, respectively, and who were the products of in vitro fertilization.

Autopsy, Brain Dissection, and Neuropathology

Autopsy was performed in the National Human Neural Stem Cell Resource laboratories under semisterile conditions. The entire brain and both globes were removed. Fresh tissues were either processed for tissue culture or cryopreserved. Tissue from the following brain areas was cryopreserved: frontoparietal cortex, centrum semiovale, hippocampus, anterior ventricular wall, caudato-putamen, and cerebellum. Substantia nigra could not be visualized because of immaturity and therefore was

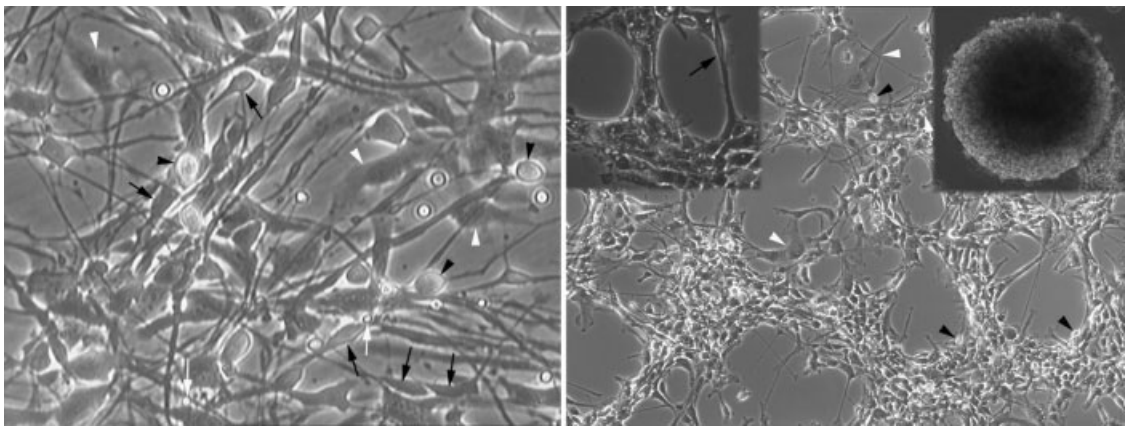

Fig. 2. Post-mortem human neural progenitor cells in culture. Cortical tissues from premature infants were dissociated and plated into medium containing EGF, FGF-2, and PDGF-AB. **Left:** Large numbers of hNPCs survived plating and remained attached to the fibronectin substrate after 24 hr. Within 1 week, cell numbers had increased so that passage was required. By 4 weeks (passage 4, shown), stably expanding populations of cells had been established. Black arrows show phase-dark bipolar cells; black arrowheads show phase-bright cells overlying the culture; white arrowheads show phase-dark pleiomorphic cells; white arrow shows very small (<5 μm) phase-bright or phase-dark profiles that were commonly seen in initial and low-passage cultures. ×20. **Right:** Agglomeration and/or sphere formation could be induced by growing cells in uncoated tissue culture flasks or by nutrient restriction for 24–48 hr. ×10. Main panel shows agglomeration of cells with **left inset** at ×20 and **right inset** showing a sphere at ×20.

not harvested from these particular brains. Retinae (not further described in this paper) and frontoparietal cortex were cultured immediately. Neuropathological examination of the brains revealed no abnormalities, gross or microscopic, in any area examined.

Primary NPC Culture

Crude cortical homogenates were plated on fibronectin-coated culture dishes, and, after 24 hr in culture, a large population of adherent cells was present on the floor of the culture dishes. At this time, the bulk of the nonadherent material was transferred to new sets of fibronectin-coated dishes. Expansion of the adherent cell population proceeded rapidly in all dishes, and the primary cultures from the first set of dishes were lifted and some of the cells plated into fibronectin-coated eight-well slides. The remaining cells were cryopreserved.

Secondary NPC Cultures

After three or four passages in GM, the NPC cultures appeared to represent a stable expanding population of cells (Fig. 2). The population was composed of cells of a variety of morphologies: Bipolar cells with long processes were interspersed with pleiomorphic cells that appeared strongly adherent to the substrate. A smaller population of small bipolar cells with short processes appeared to overlay the majority of the cells. Many single phase-bright cells, most of which appeared to be unattached or only weakly attached to the substrate, were also seen. Some of these appeared as doublets. Growth to near confluence or the lifting and replating of these cells onto uncoated plastic resulted in agglomeration of the cells and production of neurospheres (Fig. 2). Time-lapse videomicrography of the passaged adherent cultures revealed them to be highly dynamic, with high motility and extensive, evanescent intercellular contact (Fig. 3). Chromosomal analysis of passage 4 cultures after 1 week in culture revealed a normal 46XY karyotype (Fig. 4).

Expression of Markers

Under proliferation conditions, RT-PCR (Fig. 5) showed expression by cultured hNPCs of mRNA for the neurodevelopmental markers DCX, nestin, nucleostemin, and SOX2, confirming their identity as NPCs. These markers have been previously described as associated with neural stem or progenitor cells in several mammalian species (Cai et al., 2002; Tohyama et al., 1992; Tsai and McKay, 2002). Message for the EGF receptor (EGF-R; Kornblum et al., 2000) and the astroglial marker GFAP were detected as well, whereas there was no evidence for expression of the eye-associated gene products NeuroD, SIX3, recoverin, and PAX6 (Bernier et al., 2001).

Immunocytochemical analysis (Fig. 6) of proliferative hNPC cultures demonstrated extensive positivity for nestin, GFAP, Sox2, Ki67, and vimentin as well as scattered DCX-positive cells. In adherent cultures, nestin-positive and GFAP-positive cells were intermingled with some colocalization of the two antigens (Fig. 6A). In neurosphere cultures, however, the nestin-positive cells tended to segregate to the outside of the spheres and GFAP-positive cells to the inside (Fig. 6B). Almost all nestin-positive cells were also positive for Sox2, but there were some Sox2-positive cells that were negative for nestin (Fig. 6C). DCX-labeled cells were small in diameter (5–10 μm), bipolar in morphology with long processes, and tended to lie on top of the adherent monolayer (Fig. 6D), resembling, both in morphology and in location, the small, phase-bright cells seen in the living cultures prior to

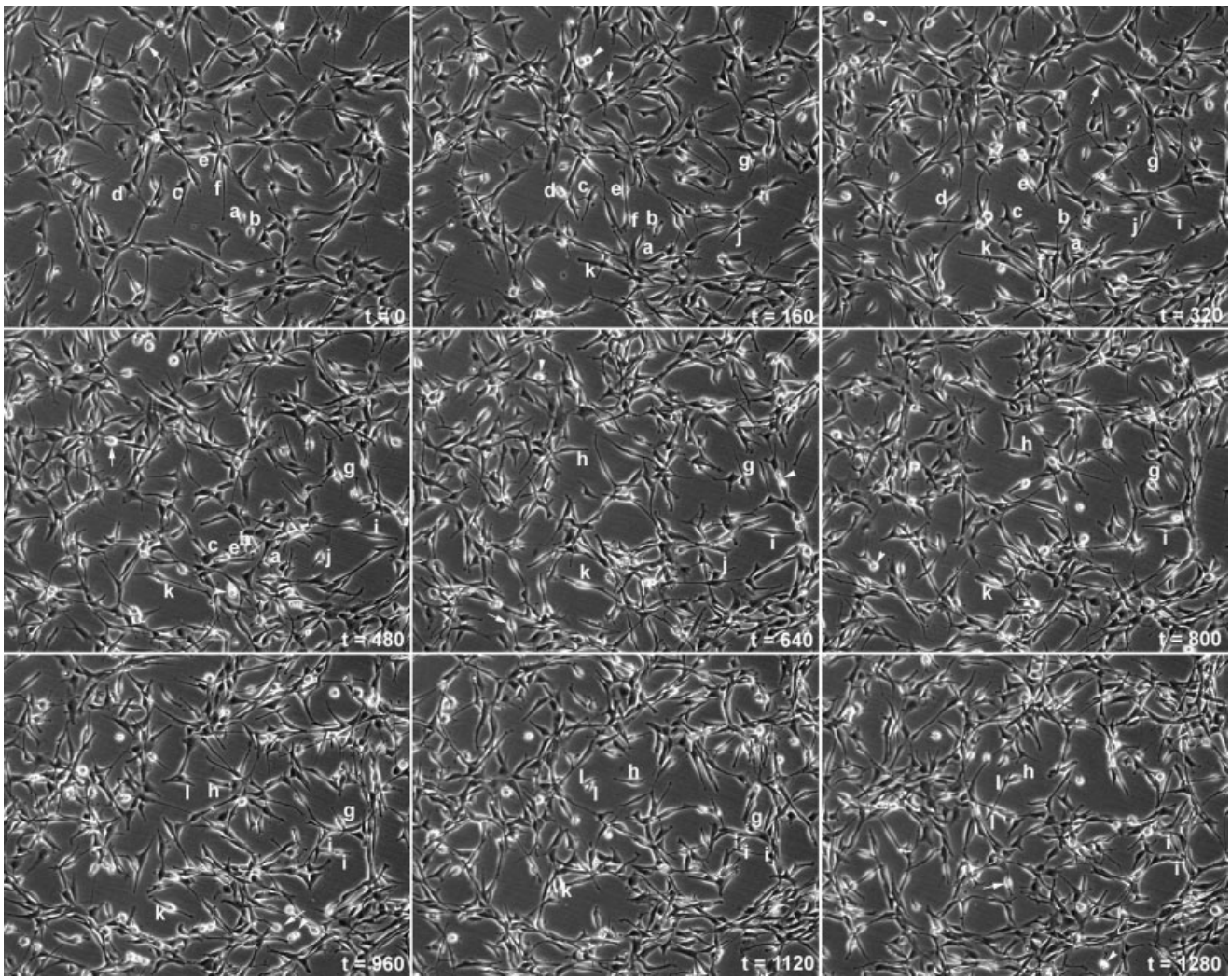

Fig. 3. Sequential images from a time-lapse photographic series. These images (×10), taken of a culture of undifferentiated hNPCs, show both the proliferation of these cells and their motility. The cells were kept humidified, at 37°C, and under 5% CO_2 for the duration of the recording. Figures, from left to right, starting at the top and descending to the bottom, represent the identical field of cells taken 0, 160, 320, 480, 640, 800, 960, 1,120, and 1,280 min after the beginning of microvideography. There is an increase in cell density in the last frame compared with the first, and many instances of cell division can be seen (arrowheads). Cells became phase bright (arrows) and retracted all processes before dividing. Cells migrated extensively and made multiple contacts with other cells; furthermore, their morphology evolved from simple to complex and back as they moved around, made contacts, and divided. Some cells in the figure have been labeled (a–l), allowing their positions and morphologies to be followed through the sequential images. An MPEG file of this recording can be found at www.nhnscr.org.

fixation, suggesting that these cells were the same population. Vimentin staining was very high in almost all cells and frequently costained with nestin (Fig. 6E). DCX never costained with nestin (Fig. 6E). Ki-67 staining revealed evidence of extensive cell division, although it rarely showed in GFAP-positive cells and was never seen in DCX-positive cells (Fig. 6F). DCX never costained with GFAP (Fig. 6F).

Flow cytometric analysis (Fig. 7) of proliferating hNPC cultures showed high positivity for MHC class I, whereas class II expression was undetectable by this method. CD14 and CD144, markers for monocytes/macrophages and endothelial cells, were also negative (Fig. 7). Cells doubly labeled with the putative stem cell markers CD15 (Capela and Temple, 2002) and CD133 (Uchida et al., 2000) show high CD133 positivity in the absence of CD15 labeling (Fig. 7). Cells doubly labeled for the neural cell adhesion molecule CD56 and the activated leukocyte cell adhesion molecule CD166 show high positivity for CD56 in the absence of labeling for CD166 (Fig. 7). A similar situation is seen for the CXCR4 chemokine receptor vs. CD166 (Fig. 7). Additional markers were also assayed, with the following results: very high CD90 (Thy-1), CD44 (Pgp-1), and CD29 (integrin β1) positivity;

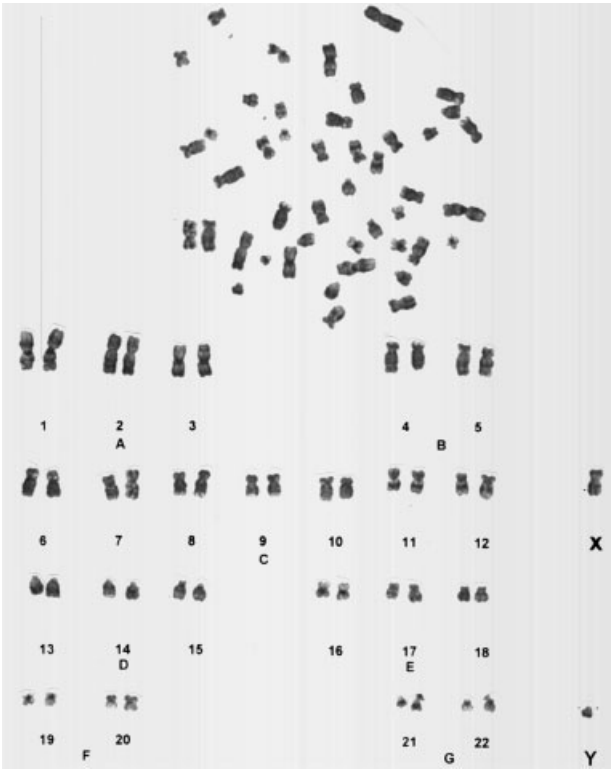

Fig. 4. Karyotype of passaged cells. Chromosomal spread of hNPC (SC23) taken during growth phase after 24 hr of colcemid treatment. The chromosome count and GTG banding patterns showed a normal 46XY karyotype.

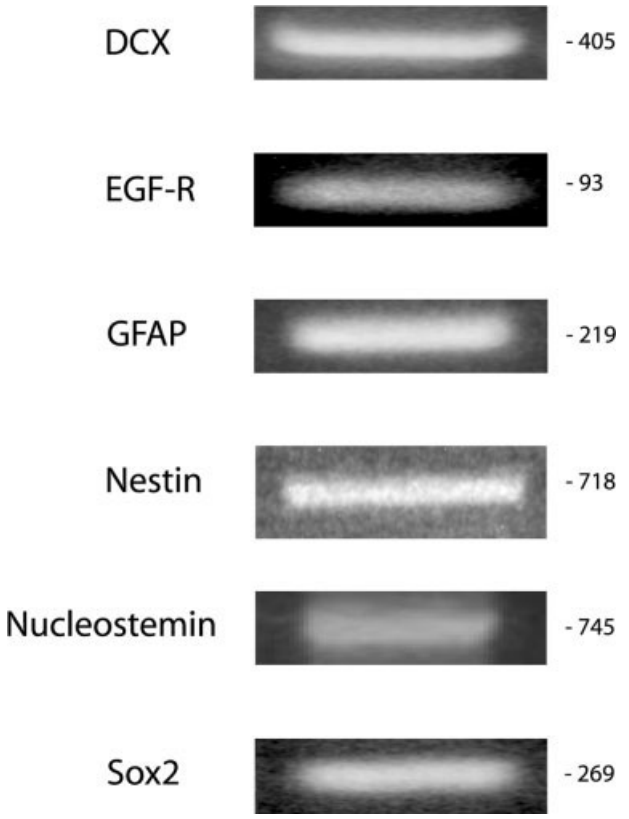

Fig. 5. RT-PCR analysis of hHPCs under proliferation conditions. Detected cDNA bands were consistent with expression of doublecortin (DCX), epidermal growth factor receptor (EGF-R), glial fibrillary acidic protein (GFAP), nestin, nucleostemin, and the stem cell-associated transcription factor SOX2. Data shown for SOX2 are from one of two different sets of primers used to assay for expression of this gene, with similar results.

some positive labeling for CD54, CD81, and CD24; little positive staining for CD45 and CD34; and negative labeling for CD106, CD9, CD26, CD49f, and CD130.

High passage cultures, under the present conditions, lost CD133 positivity and multilineage potential, as previously reported (Palmer et al., 2001; Tamaki et al., 2002; Uchida et al., 2000). In these cells, there was no change in CD15, CD106, CD34, CD14, CD144, HLA I, or HLA II staining, whereas CD9, CD81, and CD54 staining increased and CD90 and CD56 staining decreased.

Cultured hNPCs grown under differentiation conditions, consisting of growth factor removal and the introduction of GCM, low serum, all-*trans*-retinoic acid, NT3, and BDNF, expressed several neuronal markers as well as GFAP by immunocytochemistry (Fig. 6G). In addition to high levels of Tuj1 and β-III tubulin immunoreactivity, the markers NeuN and calbindin were expressed, along with FMRP, MAP2ab, Tau, and some residual DCX. Cultures differentiated with B-104-conditioned medium showed high levels of O1 staining, indicative of immature oligodendrocytes, interspersed with numerous NeuN-positive cells. The morphology of some of the O1 cells, whose processes showed a corkscrew-like appearance, was suggestive of the beginnings of myelination (Fig. 6H; Zhang et al., 2000).

Transfection

Transfection of hNPCs with a green fluorescent protein (GFP) transfection vector resulted in bright green fluorescent labeling of a subset of cultured cells (Fig. 8). Transfected cells expressed GFP for at least 2 weeks.

Electrophysiology

Whole-cell voltage-clamp recordings revealed that many of the cultured cells with neuronal morphology, grown in retinoic acid-based differentiation medium, expressed voltage-gated currents that could generate active voltage responses. Depolarizing voltage steps, in the presence of internal cesium to block potassium currents, elicited transient inward sodium currents in 60% of the cells examined (6/10; Fig. 9A). The sodium currents activate between –30 and –20 mV, peak at 0–20 mV (Fig. 9B), and are blocked by TTX (data not shown). Mean sodium current density ranged from 2 to 9 pA/pF, with a mean of 5.6 ± 0.7 pA/pF (n = 6). Voltage-gated potassium currents were present in 93% of the cells tested (14/15). Potassium currents activate at voltages above –30 mV,

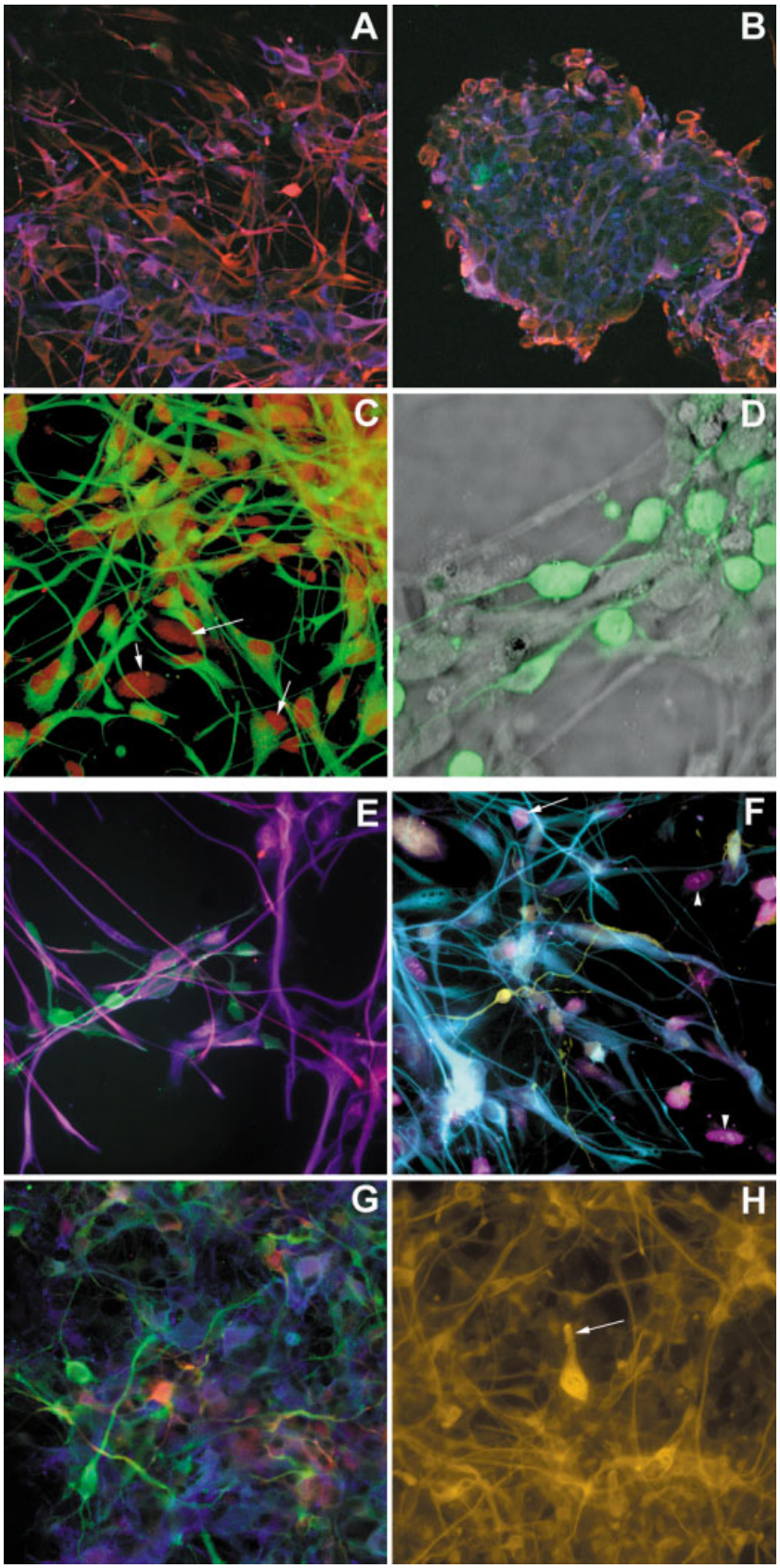

Fig. 6. Immunocytochemical staining of hNPCs. **A:** A primary culture of proliferating cells showed a high proportion of nestin (red) to GFAP (blue) staining in monolayer culture; costaining was relatively frequent. ×10. **B:** Budding neurosphere showed nestin cells (red) at the circumference, with a much higher proportion of GFAP (blue) to nestin in the interior of the sphere. ×10. **C:** Nestin (green) and Sox2 (red) staining in proliferating hNPCs. Arrows show occasional nestin-negative/Sox2-positive cells. ×40. **D:** Doublecortin (DCX) staining of hNPCs revealed a subpopulation of small, 5–10-μm-diameter, DCX-positive (green) cells against a phase-contrast background. ×40. The morphology of these is consistent with migratory neuroblasts. Both the number of these cells and their degree of immunopositivity decreased with differentiation (data not shown). **E:** Nestin (blue), vimentin (red), and DCX (green) staining in proliferating hNPCs. Nestin and vimentin frequently costain while DCX does not costain with either nestin or vimentin. ×40. **F:** GFAP (cyan), Ki67 (magenta), and DCX (yellow) staining in proliferating hNPCs. GFAP-positive cells frequently costain with Ki-67; arrowheads show GFAP-positive/Ki-67-negative cells. DCX does not costain with either GFAP or Ki-67. ×40. **G:** Cells grown for 3 weeks under retinoic acid differentiation conditions. Subpopulations stained for Tuj1 (green), GFAP (blue), and NeuN (red). ×20. **H:** Cells differentiated with B-104-conditioned medium. Subpopulations stained for O1 (orange). Note the spiral process emanating from the top of the O1-positive cell in the center of the field (arrow). ×40.

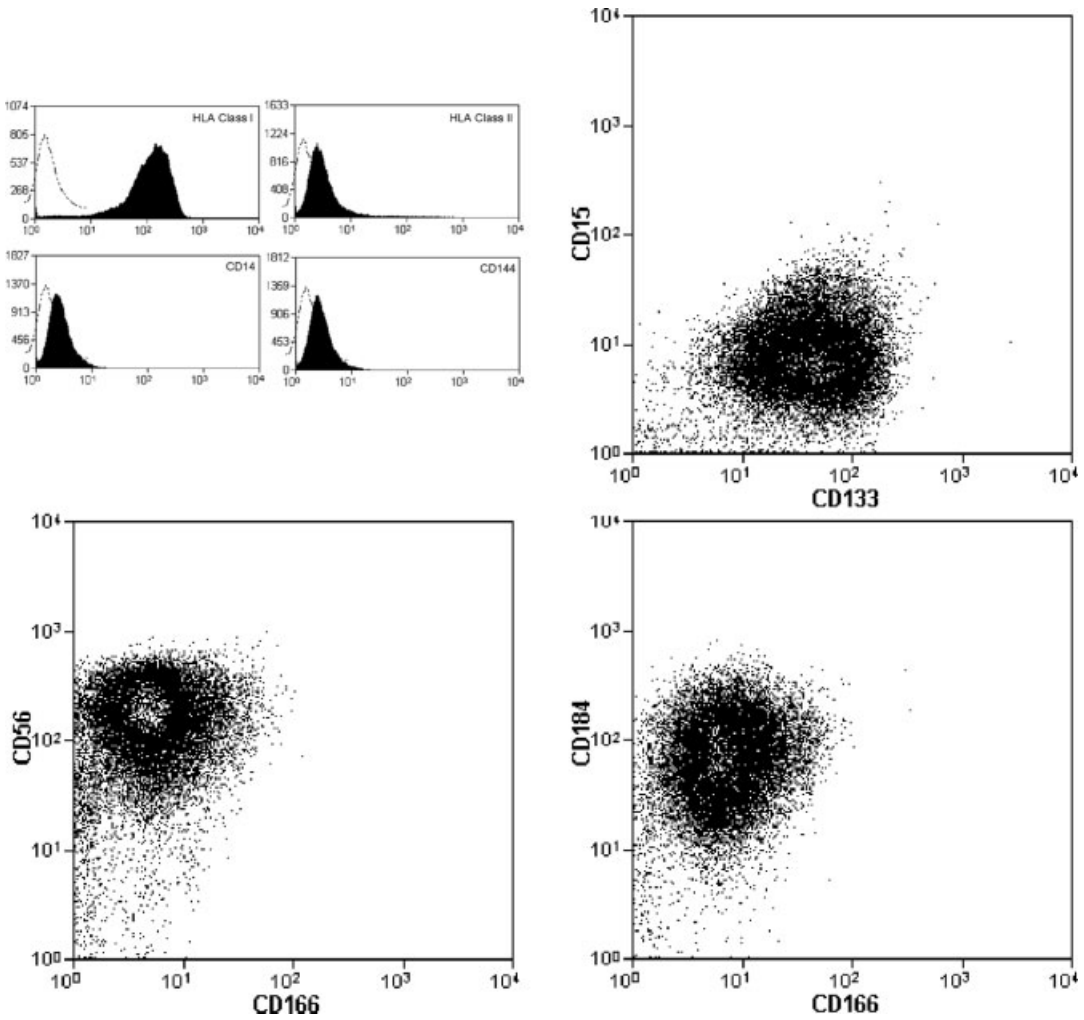

Fig. 7. Flow cytometric analysis of hNPCs. Cells were grown as monolayers under proliferation conditions and harvested for evaluation. hNPCs strongly expressed HLA class I but did not express class II at detectable levels (isotype control shown as dashed lines). Also absent were CD14 and CD144, arguing against contamination with members of the monocyte/macrophage family or vascular endothelial cells, respectively. When cells were doubly labeled with CD15 and CD133, most cells stained positively for CD133 and negatively for CD15. Cells doubly labeled with CD56 (NCAM) and CD166 (ALCAM) stained positively for CD56 but negatively for CD166, a marker present on mature neurons and fibroblasts. Cells doubly labeled with CD184 and CD166 stained positively for CD184, the CXCR4 chemokine receptor, and again negatively for CD166.

increasing in amplitude with increasing depolarization (Fig. 9C,D). In most cells, these currents showed some time-dependent inactivation during the course of a 30-msec voltage step (Fig. 9C). The peak potassium current density (determined at +50 mV) ranged from 8 to 150 pA/pF, with a mean of 43 ± 11 pA/pf (n = 14). When examined in the same cell, the peak outward potassium current amplitude was typically two- to fourfold greater than the inward sodium current. In current-clamp recordings, classic action potentials (APs) were not observed. However, the voltage response of some cells clearly deviated from a passive charging curve, with a relatively rapid rate of rise and a decay phase occurring during sustained current injection (Fig. 9D), similar to voltage responses seen in immature primary neurons (Arnhold et al., 2000; Sah et al., 1997; Vescovi et al., 1999b) with low sodium/potassium current ratios. These data suggest that these hNPCs can produce electrically functioning neurons insofar as, when differentiated, they express voltage-gated channels and voltage responses seen in immature primary neurons. Unfortunately, the immunophenotype of the recorded cells could not be ascertained.

DISCUSSION

The results of this study confirm and extend our previous results (Palmer et al., 2001) demonstrating that neural precursors can be cultured from post-mortem human brain tissue. The human neural precursors cultured in the current study were derived from the brains of developmentally immature donors; they proliferate for long

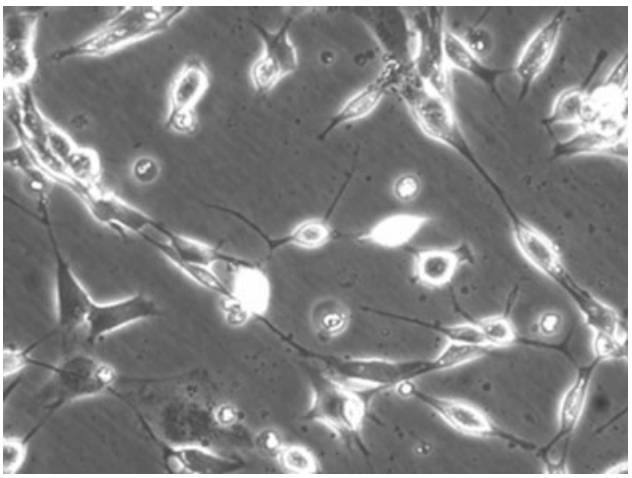

Fig. 8. Transfection of hNPCs with GFP. Human progenitor cell culture viewed 24 hr after transfection using concurrent phase and fluorescence microscopy (stimulation at 450–480 nm, detection of emissions above 515 nm). Healthy phase-bright cells were seen together with two brightly fluorescent cells in the center of the field, indicating high levels of GFP expression.

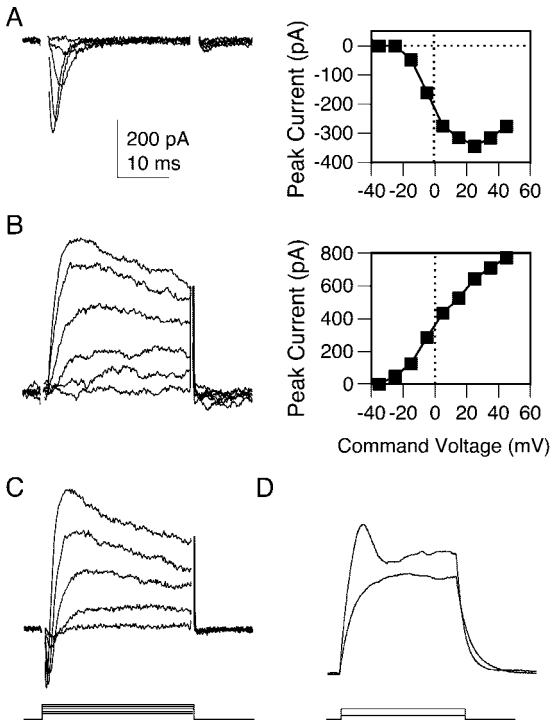

Fig. 9. Sodium and potassium currents support active voltage responses in hNPCs grown in retinoic acid-based glial-conditioned medium. **A:** Isolated sodium currents, recorded in the presence of internal cesium to block potassium currents, and the current-voltage relationship generated from this cell. **B:** Isolated potassium currents and the current-voltage relationship from this cell, which had no detectable sodium current. **C:** Transient inward sodium currents and large sustained outward potassium currents recorded in voltage-clamp. **D:** Current-clamp recordings from the same cell as shown in C. A small current step results in a passive membrane charging curve, whereas a larger current step elicits an active voltage-response characterized by an increase in rate of rise and a decay phase during the sustained depolarizing current injection.

periods in culture while maintaining a normal karyotype. They can be grown in adherent monolayers or as classic "neurospheres." In confirmation of their identity as neural precursors, cells of the cultured population expressed a number of primitive neurodevelopmental markers, including nestin, Sox2, doublecortin, and the recently reported marker nucleostemin (Tsai and McKay, 2002), as well as GFAP and the EGF-R (Imura et al., 2003). When cultured under differentiation conditions, the cells exhibited significant phenotypic changes, including the expression of the neuronal markers β-III tubulin, NeuN, and calbindin, together with the ability to generate voltage-gated currents. These post-mortem-derived cells also express a number of surface markers identified in previous work with cultured fetal progenitors, including CD133, CD56 (NCAM), the tetraspanin CD81 but not CD15 (Capela and Temple, 2002; Klassen et al., 2001), and class I but not class II MHC (Klassen et al., 2001; Uchida et al., 2000). Here we also report expression of CD24 (HSA), CD54 (VCAM), CD44 (Pgp-1), CD29 (integrin β1), and CD90 (Thy-1) in these cells as additional surface markers, which may prove useful for the bulk isolation of neural precursors from post-mortem brain tissue.

Our electrophysiological analysis suggests that these neural progenitor populations can give rise to functional neurons after differentiation. Cells with neuronal morphology expressed voltage-gated channels and immature action potentials as well as immunocytochemical markers of neurons. Although robust action potentials were not demonstrated under the differentiation conditions used, these results are consistent with other human and animal data showing that specific differentiation conditions and/or coculture may be necessary for progenitors to mature into fully functional neurons (Song et al., 2002). These conditions, specific to post-mortem human neural progenitors, are actively being sought in our laboratory.

Under standard proliferation conditions, neural progenitor cultures represent heterogeneous populations (Imura et al., 2003). For instance, in the present study, distinct subsets of cells expressed either doublecortin or the glial marker GFAP, with no evidence of double labeling. Doublecortin is expressed by migrating neuroblasts during normal development (Nacher et al., 2001) and following transplantation (Englund et al., 2002). In the cultures studied here, doublecortin-positive cells tended to be small, bipolar cells with long, thin processes and positioned above the lower-most adherent layer of cells. GFAP staining cells tended to be flattened and pleiomorphic, adhering tightly to the substrate. Examination of the time-lapse videomicrography, however, revealed a remarkable morphologic plasticity in these cultures that was not previously appreciated. Pleiomorphic cells progressively became less complex and changed, successively, into bipolar cells and

spherical phase-bright cells before dividing, the two daughter cells then becoming bipolar and, sometimes, pleiomorphic. These observations suggest that the apparent heterogeneity seen in the cultures at a single time point, such as during immunocytochemical staining, belies a flexibility in morphologic phenotype and, perhaps, therefore, in lineage potential of individual cells. Although GFAP is a well-known astrocytic marker, it is important to note that astrocytes are not necessarily postmitotic, and the relationship between astrocytes, neural stem cells, and radial glia may be more complex than previously appreciated (Doetsch et al., 1999; Imura et al., 2003; Laywell et al., 2000).

Although neural precursors may be obtained from fetal or embryonic sources, there are several advantages to obtaining cells from post-mortem tissue, depending on application. Common to all is the avoidance of the serious current ethical controversies surrounding the use of prenatal samples (Antoniou, 2001; Gershon, 2003; McLaren, 2001). In addition, both a clinical history and a pathological report are generally available in the case of post-mortem donors, providing information on genetic background as well as any developmental abnormalities or concurrent diseases, including those that do not directly involve the brain. Although surgical biopsy material may have associated with it these same data, the diagnosis of this material is, essentially, by definition, in question, thus limiting its utility. For research aimed at understanding the effects of identified genetic defects on neural development, the phenotypic expression of a particular neurogenetic disease can be ascertained with post-mortem specimens, making possible a correlation between in vitro and in vivo pathophysiology. Because of the variability in phenotypic expression of most neurological diseases, it can be problematic to make such correlations using tissue obtained at fetopsy. That is, although a fetal diagnosis may be made, trisomy 21 or Down's syndrome, for example, the eventual phenotypic expression of that diagnosis cannot be determined in utero—Down's syndrome patients express a very wide range of cognitive abilities (Rondal, 1998). This variability of phenotypic expression of a particular diagnosis is the rule rather than the exception.

Our present experience with human tissues from diverse age groups is that most samples yield viable progenitor cells for up to 36 hr post-mortem (present data, and Palmer et al., 2001). However, there are significant differences between cultures isolated from fetal compared with postnatal and adult brain. In general, fetal tissues display a higher proliferative capacity, and cell morphologies can differ from those seen in cells isolated from the neonate and adult (Palmer et al., 2001). Such intrinsic differences may prove useful in selecting cells for transplantation or other specific applications, insofar as the differences are evident even when cultures are maintained under apparently identical conditions. The specific differences of each cell population must be studied further to determine the relative merits of fetal vs. postnatal or adult-derived precursors.

As an extension of the criteria for stem cells, it is generally considered necessary to demonstrate that multipotent cells can be clonally derived from a proliferating culture. When the cells do not survive well in isolation, however, a demonstration of this kind is not necessarily feasible. This seems to be the case for a number of human progenitor types, including embryonic stem cells and hematopoietic as well as neural progenitors. Despite these concerns, there is little evidence to support the alternate theory in which long-term, multipotent CNS cultures might be composed of multiple lineages of unipotent precursors. In the CNS, at least, lineage studies strongly support the view that multipotency is preserved through most, if not all, of the period in which a progenitor is mitotically active (Sanes, 1989; Turner et al., 1990), and these findings appear to be borne out in vitro (Kalyani et al., 1997; Morshead et al., 1998; Reynolds and Weiss, 1996).

Human neural precursors clearly represent a tool with which to address many questions concerning both the development and the pathology of the human CNS, and they may provide answers not easily obtainable from studies in animals. There are many potential uses of these cells. One example is elucidation of the genetic signals involved in the differentiation of immature neural stem cells into the vast variety of fully developed cells that make up the nervous system. A full understanding of the patterns of gene expression involved in this process would greatly facilitate efforts to manipulate phenotypic plasticity to meet specific needs, be they investigative or therapeutic in nature. Another example is elucidation of the effects of genetic disease on the structure and function of these cells and the tissues into which they develop. This knowledge may provide a better understanding of CNS pathophysiology as well as potentially useful strategies for intervention. A third example is providing an in vitro system with which to screen efficiently pharmacological compounds of interest. Finally, undifferentiated human neural stem cells may provide a source of donor material for future therapies directed at developmental, degenerative, traumatic, ischemic, infectious, or neoplastic disease of the CNS. Well over 10,000 neonatal deaths, with no neurological involvement, occur annually in the United States (National Center for Health Statistics); cells harvested from these patients may ultimately open up major new options for the prevention or repair of neurological disease or injury.

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